2014

An Environmental Toxic Model of Parkinson's Disease: The Fruit Fly

Jennene Lyda
University of Montana

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By

Jennene A. Lyda

B.S Microbiology, Montana State University, Bozeman, MT
2009

Dissertation

Presented in partial fulfillment of the requirements for the degree of

Master of Toxicology

The University of Montana
Missoula, Montana

Summer 2014

Approved by:

Dr. Fernando Cardozo-Pelaez, Chair
Department of Biomedical and Pharmaceutical Sciences

Dr. Richard Bridges
Department of Biomedical and Pharmaceutical Sciences

Dr. Sarah Certel
Division of Biological Sciences

Dr. Andrij Holian
Department of Biomedical and Pharmaceutical Sciences

Lyda, Jennene, M.S., August 22th 2014
Toxicology

An Environmental Toxin model of Parkinson's Disease: The Fruit Fly

Chairperson: Fernando Cardozo-Pelaez, Ph.D.

Parkinson's disease (PD) is the second most common neurodegenerative disorder with no known specific cause; although genetic risk factors and/or environmental exposure are thought to be involved. The etiology of PD is currently unknown, although the combination of non-genetic components such as environmental exposures, the accumulation of exposure, and gene-environment interactions are thought to play a major role. However, despite this knowledge it is important to develop better models that parallel PD pathophysiology to further understand the mechanisms underlying dopaminergic neuron (DaN) damage. The use of mammalian models to study the degenerative processes in PD has been the most common approach. However, *Drosophila melanogaster* use has proven to be important to identify the physiological role of PD associated genes, and to identify pathological mechanisms of environmental toxins associated with sporadic PD. The synthetic drug MPTP (1-methyl- 4-phenyl-1,2,3,4-tetrahydropyridine) has been extensively used to generate animal models of PD. MPTP is the most used toxin model with highly reproducible effects in mice and non-human primates, and its use is a requirement for the development of new therapeutic approaches. However, MPTP neurotoxicity has not been reported in *D. melanogaster*. Results from the studies presented in this thesis show that Drosophila exposure to MPTP may be a useful model of PD, as evidenced by: loss of brain DA, reduction in tyrosine-hydroxylase positive neurons and inhibition of mitochondrial complex I. Thus, taken together this recapitulates the mammalian model

ACKNOWLEDGEMENTS

First and foremost, I would like to thank my parents for their support and unconditional love throughout my entire college career. No matter what I decided to do they always had a positive perspective and insight. Many thanks to my brother, Brent, for always telling me I was fully capable of being a scientist and encouraging me to get into this field. I would also like to thank Kelsey for the endless amount of encouragement and patience throughout my schooling, and always available to listen to scientific banter and listening intently, despite not having a clue what I was talking about.

I would also like to thank my advisor, Dr. Fernando Cardozo-Pelaez for encouraging me to enter graduate school and opening new avenues of career opportunities. Also, for him always challenging my abilities, and having a continuing excitement for science that is contagious in the lab.

I would also like to thank both the Center for Environmental Health Sciences and the Department of Pharmaceutical and Biomedical Sciences at The University of Montana, for providing a wonderful facility and funding resources for completion of experiments and my education during graduate school. I would also like to thank members of my committee Dr. Richard Bridges, Dr. Andrij Holian, and Dr. Sarah Certel for their expertise, input, and time to help me fulfill my graduate degree.

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CHAPTER ONE: INTRODUCTION

This study describes the characterization of an MPTP-toxin induced model of Parkinson's disease in *Drosophila melanogaster*, or fruit fly. The introductory chapter will: 1) Present an overview of PD and current therapies; 2) Discuss PD genetics and the etiology of the disease; 3) Explain the pathophysiology of PD and the role of mitochondria; and 4) Provide information on current models of PD and how they contribute to our understanding of PD.

Overview to Parkinson's Disease

Parkinson's Disease (PD) is the second most common neurodegenerative disease after Alzheimer's Disease (AD). Currently, seven to ten million people worldwide are diagnosed with PD with a higher percentage of patients being older than 65 years of age and this percentage is estimated to continue to rise (Parkinson's disease Foundation). Clinically, several symptoms are manifested in PD that can be classified as motor and non-motor. Motor symptoms include: resting tremor, rigidity, bradykinesia, and postural instability. These motor symptoms are the most noticeable and are diagnosed based on at least one of these symptom criteria: muscular rigidity, a resting tremor of at least 4-6 Hz, patient history, and exclusion of other disease symptoms (Worth 2013). Non-motor symptoms can accompany motor symptoms and are often the most impacting on the quality of life in patients, which include: autonomic insufficiency, cognitive impairment, and sleep disorders. Non-motor symptoms are unique in that they may be evident earlier in the disease and before motor symptoms.

Demonstrating their impact on quality of life of patients, these non-motor symptoms are the major determining factor for assisted care of patients.

The pathophysiology of this chronic progressive disease is characterized by the loss of dopaminergic neurons in the substantia nigra with the presence of Lewy bodies (LBs), which are proteinaceous, α-synuclein-containing inclusions within the surviving dopaminergic neurons (DaN). α-Synuclein is a normal endogenous protein in the brain that has a suggested role in synaptic vesicular trafficking (Overk and Masliah 2014). Although much has been done to understand the mechanisms underlying the pathophysiology, the cause of PD is still unknown. The most current hypothesis suggests that interplay between genetic predispositions and environmental exposures underlay cause on the onset of idiopathic PD.

The diagnosis of PD still relies on clinical skill of the patient's physician to make a diagnosis based on the UK PD brain bank diagnostic criteria (Dickson et al. 2009). There are currently no selective biomarkers to make a definitive diagnosis, let alone, before symptoms are overt. As mentioned before, diagnosis is based on the presence or absence of bradykinesia (the slowness of voluntary movement), and at least one of the other motor symptoms listed previously. The diagnostic standard also includes exclusion criteria (i.e history of strokes, head injury, etc.), additional supportive criteria (i.e Levodopa response, disease progression, etc.), and neuropathological presence of neuronal loss and LBs (Obeso et al. 2010). Although, despite neuronal loss and LBs having a part in

diagnosis, there is no definitive protocol for this type of assessment thus generating a need for better diagnostic imaging of the brain and biomarkers.

Currently PD is incurable and it progresses at different rates depending on the individual. There are several treatments for patients with PD, but it is important to note that these treatments only treat the symptoms of PD rather than tackling the pathophysiology causing the neuronal loss. Treatment for PD can be divided into two types based on the symptoms they are treating, motor and non-motor. The timing of initiation of these therapies is still debated. Most patients are left untreated until developing a disability that impacts their quality of life, whereas some studies report dopaminergic drug therapy before disability presence showing improvement of quality of life (Grosset et al. 2007).

The most common drug treatment for motor symptoms is Levodopa, or L-dopa. In combination with a dopamine carboxylase inhibitor, L-dopa remains the most efficacious treatment for PD motor symptoms for the last 60 years (LeWitt 2008). L-dopa is the precursor for dopamine, therefore is effective by replenishing dopamine in surviving neurons. One of the major side effects of L-dopa treatment is L-dopa induced dyskinesia's (LIDs), which involve dramatic involuntary chorea movements that can be more debilitating to patients than the motor symptoms themselves. Typically L-dopa therapy is not used in patients under the age of 50 due to a reduced response consistency over time of use, but it is still a first line of treatment in patients of all ages (Ku and Glass 2010).

Dopamine agonists (DAs) are another used group of drugs intended to activate the dopamine receptor in the absence of dopamine for treatment of

motor symptoms. These sets of drugs are typically used as a first line of defense for early PD, similar to L-dopa. Also similar to L-dopa, DAs are associated with side effects termed impulse control disorders (ICDs). ICDs occur in 17% of patients that can range in symptoms including hypersexuality, compulsive gambling, shopping and eating (Voon, Mehta, and Hallett 2011).

Monoamine oxidase B (MAO-B) inhibitors, which inhibit the enzymatic breakdown of neurotransmitter amines (dopamine), have limited use for treatment of PD but, are found to reduce mild tremor symptoms in patients. Along with the lesser-used MAO-B inhibitors, anticholinergic drugs are occasionally used for patient's symptoms predominantly having tremors, although their efficacy is hampered by its neuropsychiatric side effects in some patients. Pharmacological therapies for non-motor symptoms mentioned are minimal, thus leaving a need for development strategies to abate these symptoms.

In most patients, the combinational therapies of drugs discussed above are used for individualized treatment in patients. As the disease progresses, pharmacological therapy is less effective leaving exploration of other forms of treatment including non-pharmacological therapies. Non-pharmacological therapies include options such as deep-brain stimulation (DBS) and physical therapy. DBS is generated by trends of high-frequency electrical impulses delivered to the subthalamic nucleus or globus pallidus interna mitigating the side effects of medications or allowing for reduction of medication dose (Carron et al. 2013). Despite these treatments, there is still a need for therapies aiming for

neuro-protection and neuronal restoration, and the development of models for capturing the disease pathology and symptoms to evaluate such therapeutic approaches.

Genetics of Parkinson's Disease

Monogeneic inheritable forms of PD are thought to account for less than 10% of all cases and of these cases only a few follow Mendelian inheritance patterns. Despite representing a smaller portion of PD cases, familial inheritance studies have revealed 15 PD loci (PARK 1-15) with 11 of these PARK loci that have been described (TABLE 1). In addition to familial genes known to cause PD there are also known genes associated with sporadic PD (of no known cause) (TABLE 2) as well as variants of each of these genes that can also contribute to disease and/or individual susceptibility.

Familial PD genetics can be divided into two groups: autosomal dominant loci and autosomal recessive loci. Autosomal dominant loci include mutations in the α-synuclein gene (SNCA), PARK1 and PARK 4 and LRRK2/PARK8 (Lesage and Brice 2012). There are also other dominant genes described; yet, these are controversial and have not been replicated (*UCHL1/PARK5*), GRB10- interacting GYF protein 2 (*GIGYF2/PARK11*), *Omi/Htra2* (PARK13). The *SNCA* gene codes for a component of the amyloid precursor protein and is thought to be responsible for membrane trafficking and synaptic signaling and when

TABLE 1. 15 PD loci associated with familial inheritance

Table 1: Loci and genes associated with familial PD.

Designation	Locus	Gene	Inheritance*	Refs
Validated loci				
PARK1/PARK4	4q21	*SNCA*	AD	[5–16]
PARK8	12q12	*LRRK2*	AD	
PARK2	6q25.2–q27	*PARK*	AR	
PARK6	1p35-36	*PINK1*	AR	
PARK7	1p36	*DJ-1*	AR	
PARK9	1p36	*ATP13A2*	AR	
Other loci				
PARK3	2p13	Unknown	AD	[17–23]
PARK5	4p14	*UCH-L1*	AD	
PARK8	12q12	*LRRK2*	AD	
PARK10	1p32	Unknown	Not clear	
PARK11	2q36-37	*GIGYF2*	AD	
PARK12	Xq21-q25	Unknown	X-linked	
PARK13	2p12	*OMI/ HTRA2*	AD	
PARK 14	22q13.1	*PLA2G6*	AR	
PARK 15	22q11.2-qter	*FBXO7*	AR	

* AD: autosomal dominant; AR: autosomal recessive.

Adapted from (Coppedè 2012)

TABLE 2. Parkinson's Disease-associated genes

Locus	Gene	Inheritance	Function	Phenotype
*PARK1/4	α-Synuclein	Autosomal dominant	Involved in synaptic vesicle formation	Age of onset: 30–60 years Lewy bodies: ++
PARK2	Parkin	Autosomal recessive	An E3 ligase	Age of onset: ~30 years ‡Lewy bodies: –
PARK6	Phosphatase and tensin homologue (PTEN)-induced kinase 1 (*PINK1*)	Autosomal recessive	A mitochondrial kinase	Age of onset: 30–50 years Lewy bodies: ?
PARK7	Parkinson's disease (autosomal recessive, early onset) 7 (*DJ1*)	Autosomal recessive	Involved in oxidative stress response	Age of onset: 20–40 years Lewy bodies: ?
PARK8	Leucine-rich repeat kinase 2 (*LRRK2*)	Autosomal dominant	A protein kinase	Age of onset: 40–60 years Lewy bodies: + variable pathology
Unmapped	HtrA serine peptidase 2 (*HTRA2*, also known as *OMI*)	Autosomal dominant? Predisposition	A serine protease and/or involved in stress response	Age of onset: 44–70 years Lewy bodies: ?

*PARK1 and 4 share an entry because they have been shown to be caused by the same gene. ‡There has been one reported case of a parkin-positive patient with Lewy bodies. ++ Fulminant Lewy body pathology. + Lewy bodies present.

Adapted from (Abou-Sleiman, Muqit, and Wood 2006)

present with a mutation causes functional and structural abnormalities and the formation of LBs (Puschmann 2013). Mutations of *SNCA* can be either missense or point-mutations with a mutation frequency of about 1% (Irwin, Lee, and Trojanowski 2013). Phenotypically, *SNCA* mutations resemble sporadic PD patients with the presentation of earlier onset and atypical features, including cognitive decline, psychiatric problems and autonomic dysfunction (Singleton, Farrer, and Bonifati 2013).

Another common autosomal dominant mutation is in *LRRK2*, which is associated with young-onset familial (~10%) and late-onset sporadic PD (~2%) (Krüger 2008). LRRK2 protein's role in dopaminergic cell death is unknown but it is thought to be due to a decrease in its kinase activity (Wickremaratchi, Ben-Shlomo, and Morris 2009).

Genes associated with autosomal recessive forms of PD include: Parkin: *PARK2*, PTEN-Induced Putative Kinase 1 Gene (*PINK-1*): *PARK6*, *DJ-1*: *PARK7*, and ATP13A2 Gene: PARK9. Typically, PD cases due to these genes appear in offspring of unaffected parents due to their inheritance pattern, therefore sometimes determined sporadic due to its lower frequency (Lin and Farrer 2014). Typically these alleles result in loss of function due to inactive protein and/or absence of protein synthesis.

Parkin mutations account for the majority of autosomal recessive cases in patients and there are approximately 170 different mutations possible on the chromosome (Mata, Lockhart, and Farrer 2004). Parkin mutations are characterized by early onset of disease without the presence of LBs. Parkin is

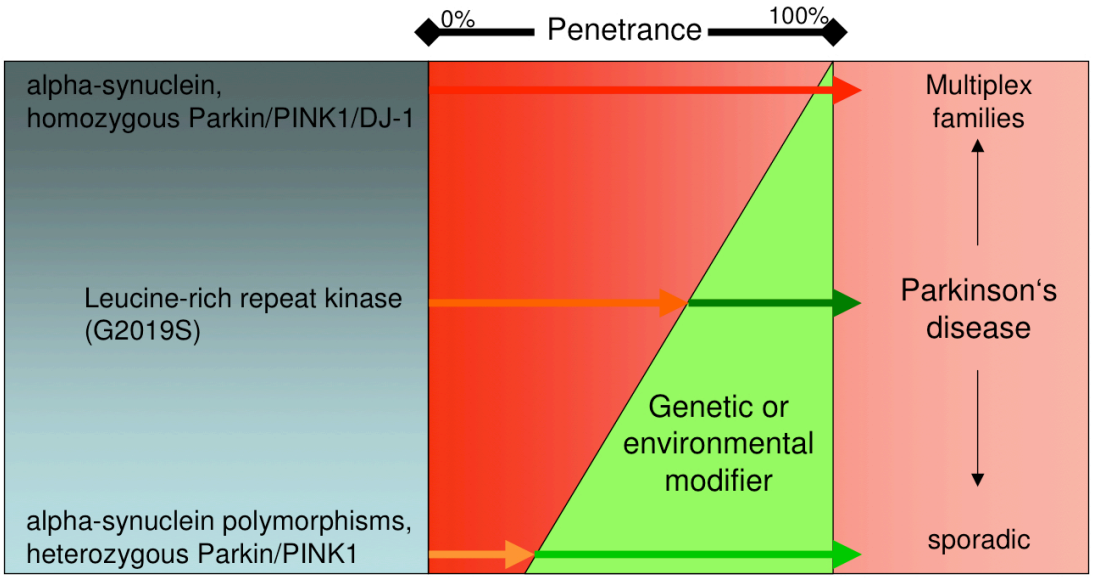
thought to be responsible for mitochondrial-induced apoptosis through unregulated release of cytochrome c.

PTEN-induced putative kinase 1 (PINK-1) is also an autosomal recessive mutation on a different chromosome from the Parkin mutations. The PINK-1 mutations are responsible for mitochondrial deficits, therefore leading to mitochondrial dysfunction. Typically, PINK1 is involved in mitochondrial maintenance participating in an upstream pathway in mitochondrial autophagy (Kawajiri et al. 2011). Similar to PINK-1, which responds to mitochondrial alterations, DJ-1 (PARK7) responds to changes in oxidative stress environment for mitochondrial protection. This response action causes DJ-1 to bind to PINK1 to promote degradation by the ubiquitin proteasome system (UPS) by damaged parkin proteins. Mutations in the ATP13A2 Gene (PARK 9) lead to pathologies that resemble idiopathic PD. The protein coded by PARK 9 is thought to be involved in homeostasis of manganese (Chesi et al. 2012). The other PARK loci are thought to be mainly involved in the UPS system and disruption of this protein homeostatic pathway.

Overall, genetics play an important role in PD pathology and have helped our understanding of the pathological pathways linked to the neurodegenerative process. As shown in Figure 1, the interplay between environment and genetics is the basis of the current hypothesis of etiology of PD pathology. However, whether it is a specific environmental agent or family of agents; as well as which genes are more relevant for the development of the idiopathic form of PD needs to be fully determined. In addition to genetics, epigenetic mechanisms are

thought to have a key role in modulating the risk of PD development; this is supported by findings of differential methylation patterns in some of the genes discussed above (e.g LRRK2, SNCA).

FIGURE 1



Adapted from (Krüger 2008)

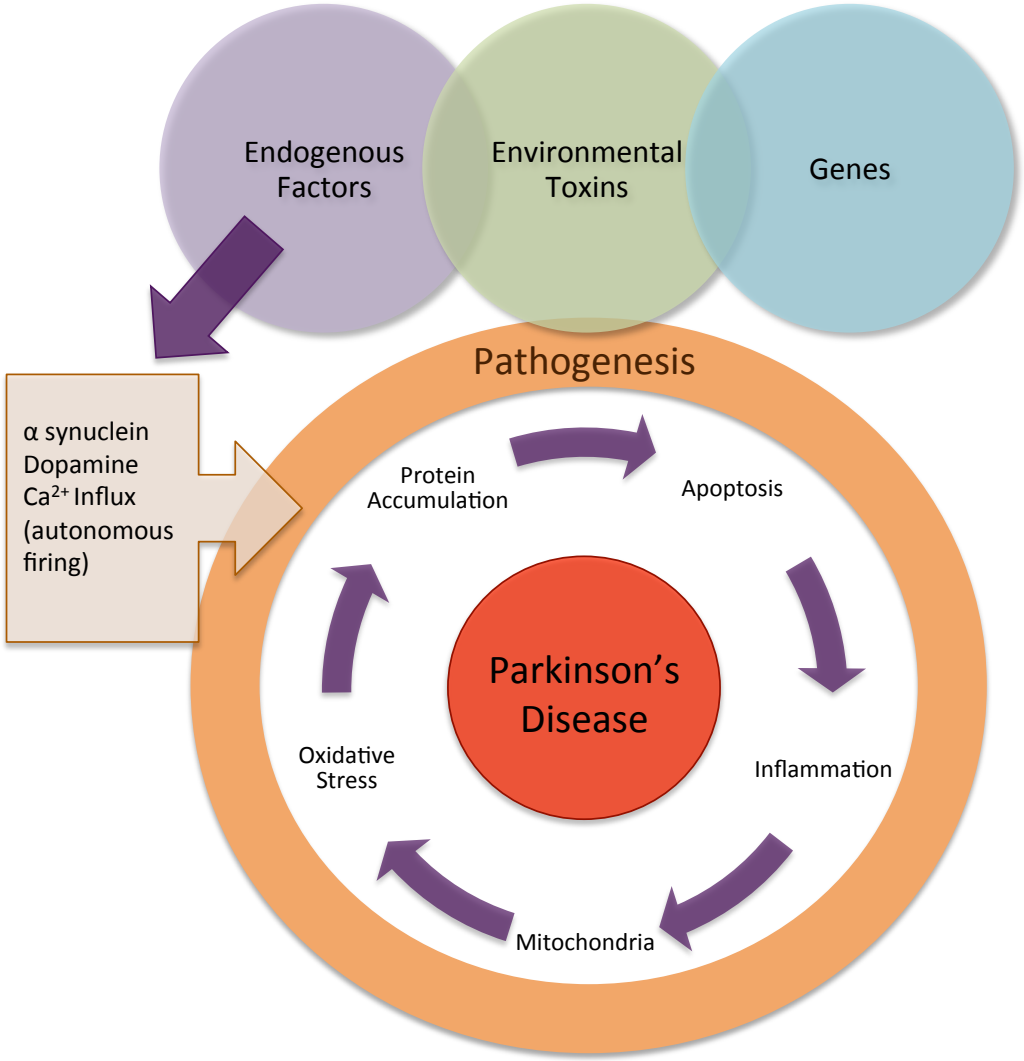
Figure 1. Penetrance of PD and susceptibility factors

Etiology of Parkinson's Disease

The exact etiology of PD is unknown; however, there are hypothesis about what contributes to the different forms of the disease. One of the hypotheses suggests a major genetic component for the development of early-onset forms, while the late-onset forms are mostly due to environmental factors. It is believed that the link between these two types of hypothesis is at the root of the cause and the mechanism linked to the dopaminergic neuronal death. Three common endogenous factors, dopamine, alpha-synuclein, and calcium, are linked to five different mechanisms of DaN degeneration. The first mechanism involves generation of oxidative stress through the oxidation of cytosolic dopamine. The second mechanism is initiated by the release of dopamine due to synaptic vesicle permeabilization from mutations and/or excessive alpha-synuclein. The third mechanism is through dopamine binding to alpha-synuclein protofibrils, allowing them to be a persistent toxic species. The fourth proposed mechanism is driven by increased intracellular calcium leading to the dysfunction of the mitochondria. The fifth mechanism is the inhibition of the lysosomal degradation.

Figure 2 illustrates the possible interactions between endogenous factors, environment, and genes, with the possible outcomes leading to pathology seen in PD. This best summarizes the most current hypothesis for the etiology of PD, taking into account all these factors and how they may interplay. Thus, determining a direct explanation or identifying a single cause, is a complicated endeavor.

FIGURE 2



Adapted from (Fahn 2010)

Figure 2. Etiologic Factors Involved in Parkinson's disease

Role of Mitochondria in Dopaminergic Cell Death

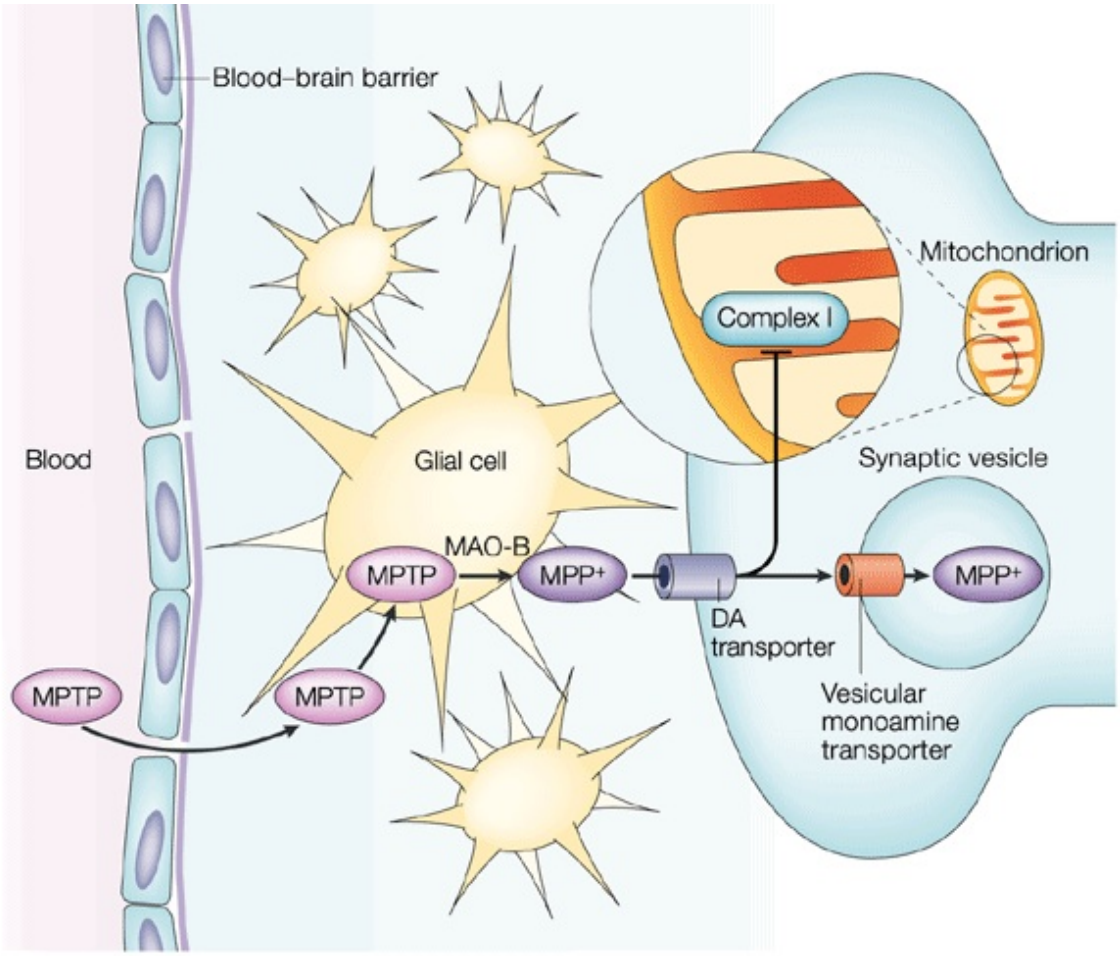
Mitochondrial dysfunction has been implicated as a key component to PD pathogenesis. Additionally, the discovery of toxins such as, MPTP (1-methyl- 4-phenyl-1,2,3,4-tetrahydropyridine) has further implicated the role of environmental toxins and that of mitochondria dysfunction in PD. The link between mitochondria dysfunction and PD was first suggested when it was shown that patients with PD had decreased complex I activity in the substantia nigra, skeletal muscle and platelets (Mizuno et al. 1989), and further supported by identifying that the major mitochondrial Complex I inhibition is a common mechanism linked to dopaminergic toxins (Banerjee et al. 2009).

MPTP enters the brain by crossing the blood-brain barrier where it is converted to MPP+ by the enzyme monoamine oxidase B. MPP+ then binds to the dopamine transporter and is transported into neurons. Inside the neurons MPP+ has a high affinity for the complex I of the electron transport chain (ETC), inhibiting respiration. This inhibition of the ETC induces a build-up of free radical production, oxidative stress, decreased ATP production, subsequent release of intracellular calcium, and excitotoxicity. Furthermore, it has been shown that MPP+ releases dopamine leading to increased oxidative stress (Carta, Carboni, and Spiga 2013).

The complete picture of mitochondrial involvement with PD began after the discovery of familial genes of PD. As mentioned in the section discussing genetics and PD, many of the familial genes associated with PD have some role

in physiological pathways that directly or indirectly are associated with the mitochondria; thus, inferring that normal mitochondria function is a major factor in promoting DaN viability (Chesselet and Richter 2011). Figure 3 depicts the accepted mechanism for dopaminergic neurotoxicity associated with MPTP, while Figure 4 illustrates the purported role in mitochondrial dysfunction and oxidative stress of some of the genes implicated in familial forms of PD.

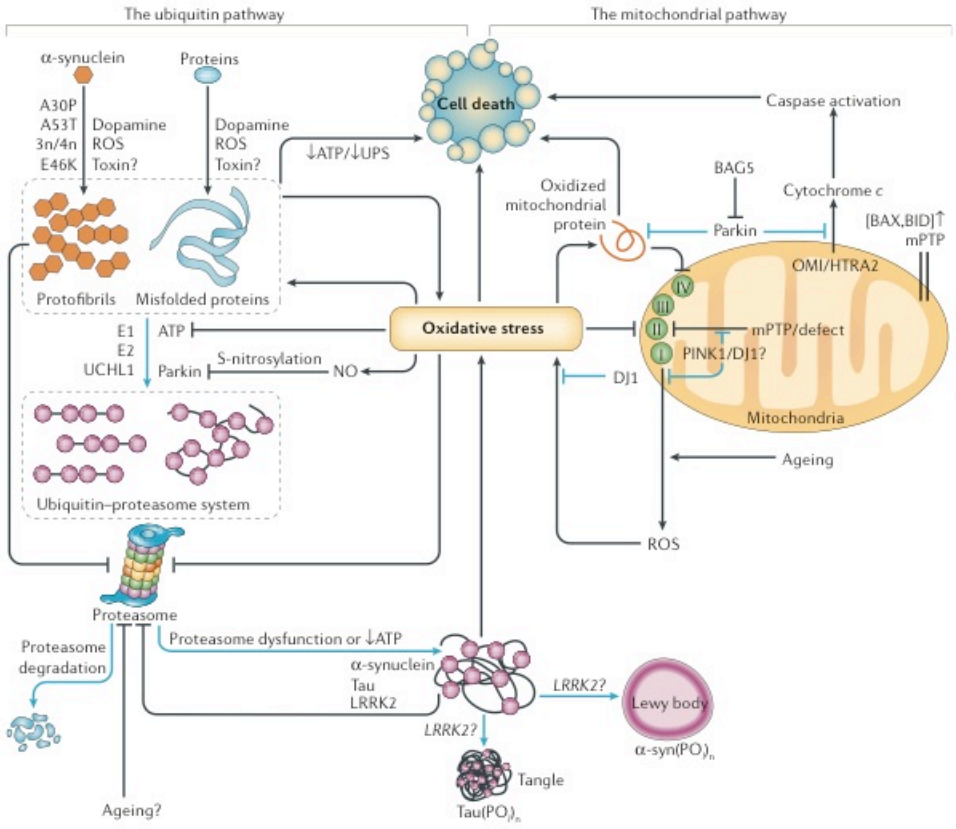
FIGURE 3



Adapted from (Vila and Przedborski 2003)

Figure 3. MPTP Inhibition of Mitochondrial complex I

FIGURE 4



Adapted from (Abou-Sleiman, Muqit, and Wood 2006)

Figure 4. Genes Involved in Mitochondrial Dopaminergic cell death

Toxin-Induced Models of Parkinson's Disease

The current knowledge and advancements in the field of PD can be attributed to the use and discovery of PD models. Although there are many models available, as will be discussed, there are unique characteristics linked to toxicological mechanism, exposure, and progression of damage that would make a model more suitable than others to answer a specific question (Beal 2001). First, the main characteristic of PD pathology is the gradual loss of dopaminergic neurons and the age-dependent formation of LBs. For any model, a loss of dopamine of about 50%, should be detectable by biochemical and neuropathology. Second, the model should link dopamine loss and motor-deficits; thus, reenacting the classical hallmarks of the disease (bradykinesia, resting tremor, and postural rigidity) (Blesa et al. 2012). Finally, tying the model to a single mutation is important to discover disease etiology. Currently, there is not a single model that combines the major risk factors of PD. A generation of such model may allow for shorter time for evaluation of experimental therapies and to assess the effect of the aging process.

The use of dopaminergic toxins in vertebrates is the most common approach to model the neuronal loss in PD. Toxin-induced models include: 6-hydroxydopamine (6-OHDA), rotenone, and MPTP. Figure 5 depicts the most accepted mechanism for each of these toxin-induced models. 6-OHDA is used in rats, mice, and primates by direct injection into the substantia nigra, usually generating a unilateral lesion (Harvey, Wang, and Hoffer 2008). Although, LBs

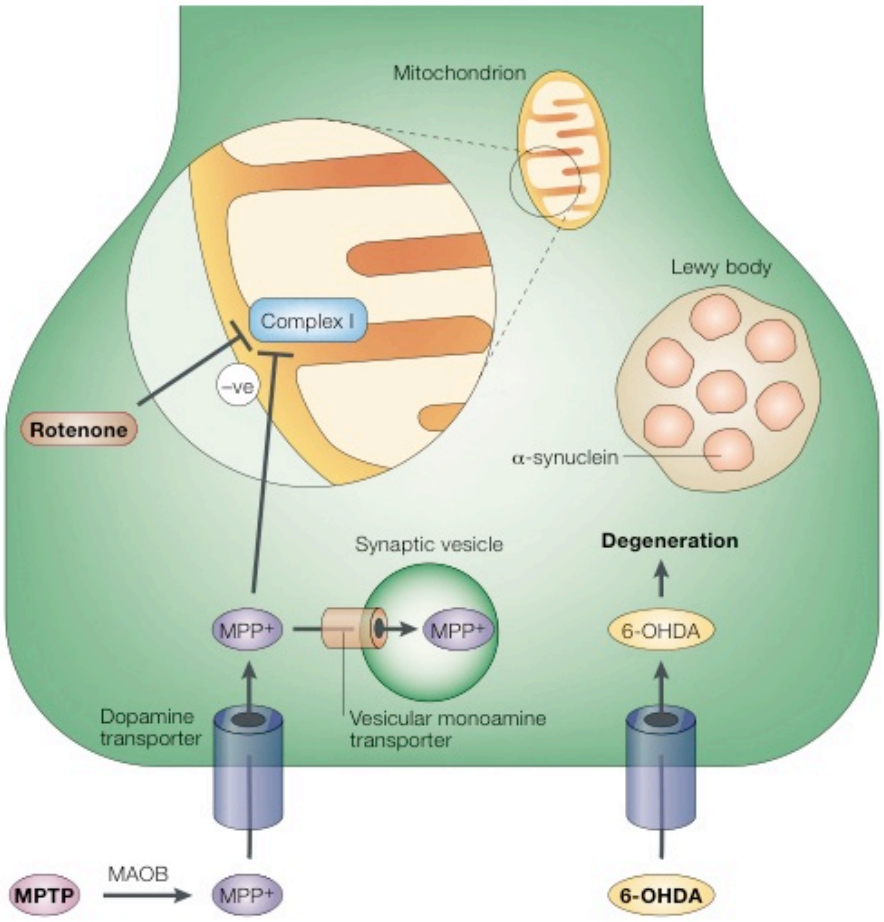
are not present, there is a loss of targeted DaNs that can lead to a reduction in motor abilities (Tieu 2011).

Rotenone is a pesticide that is still used in the fishing industry today. Rotenone is a selective inhibitor of mitochondrial complex I. Chronic treatment with rotenone has replicated the specificity of dopaminergic nigral neuron loss in rodents, and is the only toxicological model to generate LBs. This model, however, is not widely used due to the variability and lack of consistency of the neuropathological changes (Lapointe et al. 2004)(Zhu et al. 2004).

MPTP is the most common used toxin to generate a PD model. As discussed in the previous section, it was one of the first models to link the inhibition of mitochondria complex I and PD. Although several animal species have been treated with MPTP to recapitulate the model (sheep, dogs, guinea pigs, cats, mice, rats, and monkeys), the MPTP-monkey model is still the standard for testing of therapeutic interventions (Carta, Carboni, and Spiga 2013). Both monkeys and mice treated with MPTP have selective progressive loss of nigrostriatal DaN, reproducible motor deficits, but no LBs (Tieu 2011).

Table 3 (excluding paraquat/maneb) summarizes the current toxin-induced models and what characteristics are present in each one. Having these models as the current standards has been beneficial, although the need for a better model that is cheaper, faster to screen therapeutic interventions, and accessible is necessary.

Figure 5



Adapted from (Abdel-Aal, Assi, and Kostandy 2011)

Figure 5. Mechanisms of Toxin-Induced Models of Parkinson's disease

Table 3. Toxin-Induced Models of Parkinson's disease

Models	Pathology					Behavioral phenotypes	
	Nigrostriatal damage						
	SN cell body	Str. terminals	Str. DA	Extranigral pathology	LB	Motor (L-dopa or apomorphine responsive)	Nonmotor
6-OHDA							
Rat							
Stereotactic injection to SN, MFB, striatum	Yes	Yes	Yes	No	No	Yes	Cognitive, psychiatric, and GI disorders
MPTP							
Nonhuman primates							
i.p, i.m, intracarotid infusion	Yes	Yes	Yes	LC	Yes	Yes	Numerous (see Fox and Brotchie 2010; Bezard 2011)
Mouse							
Acute, subacute (i.p.)	Yes	Yes	Yes	No	No	Yes	Transient ↑colon motility
Chronic (osmotic minipumps)	Yes	Yes	Yes	LC	Yes	Yes	ND
PQ							
Mouse							
i.p.	Yes	Conflicting	No	LC	Yes	ND	ND
PQ/Maneb							
Mouse							
i.p.	Yes	ND	Yes	ND	Yes	ND	ND
Rotenone							
Rat							
Infusion via osmotic minipumps	Yes	Yes	Yes	ND	Yes	Yes	↓GI motility
i.p. injection	Yes	Yes	Yes	GI	Yes	Yes	↓GI motility

Abbreviations: SN, substantia nigra; Str., striatal; LB, Lewy bodies; LC, locus coeruleus; GI, gastrointestinal; MFB, medial forebrain bundle; ND, not determined.

Adapted from (Tieu 2011)

The use of Drosophila melanogaster as a neurodegenerative model

Using *Drosophila melanogaster* as a model for neurodegenerative processes has become increasingly popular, especially in the field of PD. The current models of PD do not include all the major risk factors linked to the pathological processes observed in the disease, whereas *Drosophila* models are amiable to incorporate most of them and may fill this need (Guo 2012). The use of an invertebrate model has many advantages over traditional vertebrate models, such as: short lifespan, ease of genetic manipulation, and a conserved DA neurotransmitter system. The drosophila genome has limited redundancy and is 77% homologous to humans (Rubin 2011). Therefore, studying familial genes associated with PD (DJ-1, PINK1, PARKIN, LRRK2, and VPS35) and their role in pathogenesis in the fly is possible.

In addition to genetic forms of PD, toxin-induced models of PD in *Drosophila* are becoming useful tools. Several studies have looked at rotenone and paraquat (PQ) (a proposed mitochondrial complex I inhibitor) in *Drosophila* to investigate susceptibility of PD genetic models and its role in neuronal cell death (Botella et al. 2009). Not only do these models induce loss DaNs, but also, there is evidence of behavioral and histological changes, completing the pathological picture of PD (Trinh et al. 2010). Despite studies in *Drosophila* examining rotenone and paraquat, as models, there are no studies with MPTP in drosophila.

The hypothesis tested and presented is that wild-type *Drosophila melanogaster* will be susceptible to MPTP and will generate a robust model with classical signs of PD pathology as defined by loss of dopamine, tyrosine hydroxylase-positive neurons, and motor behavior deficits. Therefore, we want to determine how MPTP, implicated in other PD models, impacts pathophysiology and toxicity in *Drosophila* in the following two aims:

Specific Aim 1: To define the time- and dose-course susceptibility to MPTP dopaminergic neurotoxicity in *Drosophila*.

Specific Aim 2: To correlate inhibition of mitochondrial Complex I with loss of the dopaminergic system in *Drosophila*.

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CHAPTER TWO: AIM ONE

Defining the dose susceptibility to MPTP dopaminergic neurotoxicity in *Drosophila melanogaster*: Analysis of PD pathological markers after toxin exposure

Abstract

Drosophila melanogaster has become a useful tool for assessing models of PD. The use of toxin-induced models, such as rotenone and paraquat, has been investigated in *Drosophila*, but MPTP has not been tested. This chapter describes a method for MPTP exposure in *Drosophila* and investigates biochemical and pathological markers associated with PD. Exposure to MPTP reduced dopamine in brains of male flies and altered behavior assessed by startle-induced negative geotaxis.

Introduction

Models of Parkinson's disease (PD) are important in the discovery of the pathophysiology of the disease. Although there are several animal models looking at familial genetic mutations that have roles in the disease pathways, models portraying idiopathic PD, which are the majority of disease cases, are critical in discovery of mechanisms.

Drosophila melanogaster (fruit fly) has been increasingly useful in PD modeling. More specifically, *Drosophila* has been used for investigating rotenone and paraquat (PQ), both of which are commonly used for modeling idiopathic PD. Recently, rotenone has been used in several studies examining PD pathology markers including: histology and behavioral assessment (Hosamani, Ramesh, and Muralidhara 2010). PQ exposure in *Drosophila* has also been established in some studies for acute and chronic exposure (Rzezniczak et al. 2011) (Lawal et al. 2010). Despite both of these models showing pathologic markers seen in PD, there are several inconsistencies with both the rotenone and paraquat models in other animals used (Vila and Przedborski 2003). These types of inconsistencies include: low percentage of rats exposed showing nigrostriatal lesions, rotenone is specific to rats and does not work in other animal models, and there are no reported human toxicity exposures resulting in PD (Nussbaum and Polymeropoulos 1997). Similarly, results with paraquat, remain inconsistent due to conflicting reports in relationship to the susceptibility of dopaminergic neurons after exposure, or whatever PQ combination with maneb (manganese ethylenebisdithiocarbamate) is a more effective model (Thiruchelvam et al. 2000)

(Thiffault, Langston, and Di Monte 2000). MPTP has been used in animal models of genetic forms of PD, contributing to the identification role(s) these genes play in pathophysiology. Therefore, the most common and highly researched and reproducible toxin-induced model, MPTP, should be developed in the fruit fly.

The following study describes an analysis of PD pathological markers in *Drosophila* males after MPTP exposure. Although *Drosophila* has been used as a model for rotenone and paraquat toxicity, the MPTP model has yet to be investigated. Optimization of the CAFÉ assay with MPTP was achieved by spectrophotometry and measurement of consumption. The method described here was used to analyze dopamine in brain of flies exposed to increasing concentrations of MPTP and to assess behavior and tyrosine hydroxylase expression after exposure. Our results indicate that acute MPTP exposure leads to a dose-dependent loss of dopamine, a trend in reduction of TH-positive neurons and alterations in motor behavior in flies. Thus, these results indicate that the MPTP toxin-induced model in Drosophila melanogaster is a useful tool to use for pathophysiology in PD.

Materials and Methods

Chemicals and Reagents

All reagents were purchased from Sigma Aldrich unless otherwise indicated (St Louis, MO). MPTP were made fresh for each exposure with 5% sucrose w/v.

Drosophila Stocks, Husbandry, and transgenics

D. melanogaster wild-type (Canton-S) was obtained from the Bloomington Stock center (Bloomington, IN). The flies were reared on agar medium (1%, w/v brewer's yeast; 2%, w/v sucrose; 1%, w/v agar; 0.08%, v/w Tegosept®) at constant temperature and humidity (23°C; 60% relative humidity, respectively). The flies were reared in 16 x 100 mm vials containing approximately 5 mL of medium at constant temperature, humidity (60%) and under 12h dark/light cycle. All experiments were performed with the same WT Canton-S strain except for Immunohistochemistry.

Crosses to generate the TH-Gal4:20xUAS-6xGFP (green fluorescent protein) flies were set up under standard conditions at 25° C. Male flies containing the TH-Gal4 UAS transcript were mated with females containing the 20x-6xGFP insert, then pupae were collected into isolation vials.

Drosophila MPTP exposure

The capillary feeder method (CAFE) CAFÉ assay used for exposure was adapted from JA, et al. 2007 (Ja et al. 2007). The exposure model used was similar with two chambers. The inner chamber, containing the flies, was composed of a 25 cm vial with perforated lid for air and water exchange from the

outer chamber, a 1000 mL beaker with 20 cm distilled water, and additional holes for capillary tube insertion. Capillary tubes at 40 cm length from World Precision Instruments (Sarasota, FL) (cat. #4878) were filled by capillary action with liquid and inserted into the inner vial lid with an additional layer of mineral oil to prevent evaporation. All exposures (MPTP or sucrose control) were conducted for 24 hours in a 25°C room. After each 24-hour exposure, flies were placed back into isolation vials containing normal food medium for 24 hours before analyses. All exposures were done on 5-10 day old males including flies for immunohistology.

Brain dopamine via HPLC-ED

For sample preparation, fifteen 5-10 day old adult male flies were anesthetized with CO_2, and then transferred to cold phosphate buffer saline (PBS) preceding dissection. Brains were dissected in cold PBS by gentle forceps manipulation under a dissecting microscope, and then transferred to a 1.5 ml microcentrifuge tube containing 100 µL perchloric acid (0.05 M) with 30 ng/mL DBA (internal standard). On ice, brains were homogenized using a hand-held sonicator and centrifuged for 15 minutes at 4°C at 14,000xg.

For reverse-phase high liquid chromatography (rp-HPLC) measurement of dopamine, a modified rp-HPLC protocol for catecholamine measurement was used as described earlier (F. Cardozo-Pelaez et al. 1999) (Fernando Cardozo-Pelaez, Cox, and Bolin 2005). Chilled fly brain homogenates were centrifuged and 50 µl of supernatant fluid was eluted through a 250 × 4.6 mm C18 column (Agilent Technologies Santa Clara, CA). The mobile phase consisted of water:acetonitrile (9:1, vol/vol) containing 0.15 M monochloroacetic acid, 0.12 M

sodium hydroxide, 0.60 mM EDTA, and 1.30 mM sodium octyl sulfate. The flow rate was kept at .8 mL/min (ESA Model 582 Solvent Delivery Module, Chelmsford, MA) and the column eluent was analyzed with an electrochemical detector (ESA Model 5600A CoulArray Detector, 3 ESA Model 6210 four channel electrochemical cells, Chelmsford, MA -50, 0, 100, 200, 300mV). All RP-HPLC data were recorded stored and analyzed using CoulArray for Windows 32Software (ESA Chelmsford, MA). DA was monitored at 300mV. The ratios of peak height measured in Nano amperes (nA) produced by DA to the peak height (nA) produced by DBA (internal standard) in the samples were used to obtain the analyte levels from a calibration curve. Data was expressed as micrograms of analyte per brain (µg/brain).

Immunohistology & Confocal Imaging

For immunohistochemistry; after cuticle was removed, the brains were fixed in 4% paraformaldehyde. Confocal microscopy image processing was performed using a Olympus IX81 confocal microscope and Fluoview FV1000 viewer and application software. Antibody dilutions were as follows: rabbit anti-Tyrosine Hydroxylase 1:200 (Abcam) and Goat anti-rabbit 594 1:200 (Abcam). Images were acquired at a resolution of 1024 pixels. The counting of DA neurons labeled with TH:GFP and 594 labeling were tracked through confocal Z-stacks.

Startle-Induced Negative Geotaxis

To assess locomotor climbing activity, we transferred into empty plastic vials 30-50 male flies that had been exposed to MPTP or 5% sucrose were transferred into empty 50 mL conical tubes. Flies were allowed to acclimate in the vial for 15

minutes before the test began. The vials were tapped to startle all flies to the bottom and then the number of flies climbing up to the top quarter (7cm) of the vial within a 15 second period, were counted. Each vial was used for three consecutive measurements, allowing for 5 minutes rest period, with a total of ~100 flies per treatment group assessed. This method was designed similarly to previous behavioral tests (Ali et al. 2011) (Nichols, Becnel, and Pandey 2012).

Statistical Analysis

All data were analyzed using Microsoft Excel and Prism 5 software. The t-test was implemented when comparing just two data groups where significance was p-value > .05. For comparison of multiple groups, ANOVA was used to determine significant differences between each group with significance of p-value >.05.

Results

Establishment of the CAFÉ assay

The first set of experiments were designed to test whether flies would consume MPTP hydrochloride dissolved in 5% (w/v) sucrose from the capillary. Typically, fruit flies are attracted to sugar containing medium, therefore it remained a question to whether there would be an aversion and preference for just the sucrose control compared to sucrose containing MPTP hydrochloride. First, green food color was added to each sucrose or MPTP solution in capillary tubes for the 24 hour exposure period. We previously, by spectrophotometry, identified the maximal absorbance by spectrophotometry for the green food coloring added (Figure 6). Using the maxima wavelength (630 nm), we compared the amount of liquid intake between flies exposed to sucrose or sucrose/MPTP mix. Figure 7 shows evaluation of liquid intake, as determined by the amount of green color present in sucrose alone (Figure 7A) or sucrose/MPTP mix (Figure 7B) and quantitative assessment by absorbance at 630 nm of extracts from whole flies after exposure to 5% sucrose or 12 mM MPTP (Figure 7C). There is no visual or quantitative difference in liquid consumption. A secondary assessment was done by measuring amount of liquid consumed in a period of 24 hours at 0, 24, or 36 mM MPTP. Lack of statistical difference among the groups (Figure 8) indicates that the level of MPTP is not adverse to the *Drosophila melanogaster*

FIGURE 6

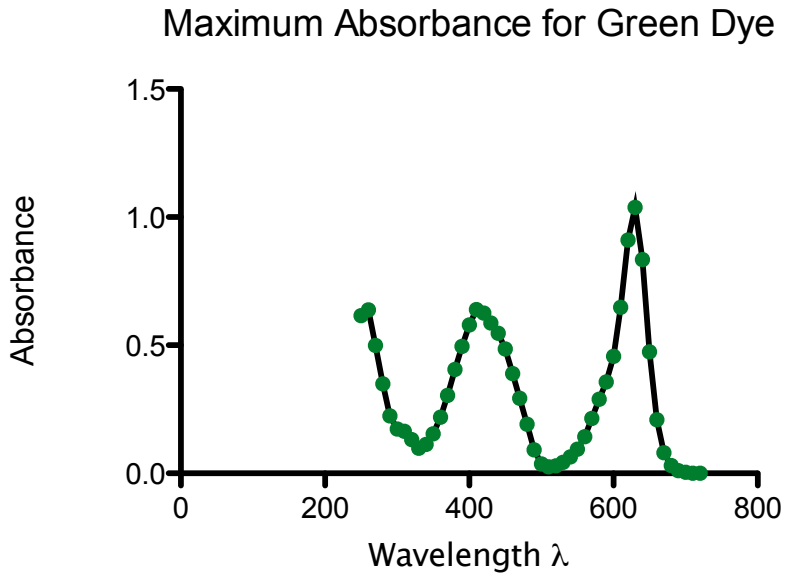


Figure 6. Visual spectral analysis of Green Dye

Maximum absorbance was determined for green dye. Each absorbance was measured by spectrophotometer to determine the wavelength for maximum absorbance. 630 nm was the maximum to be used for further consumption analysis.

FIGURE 7

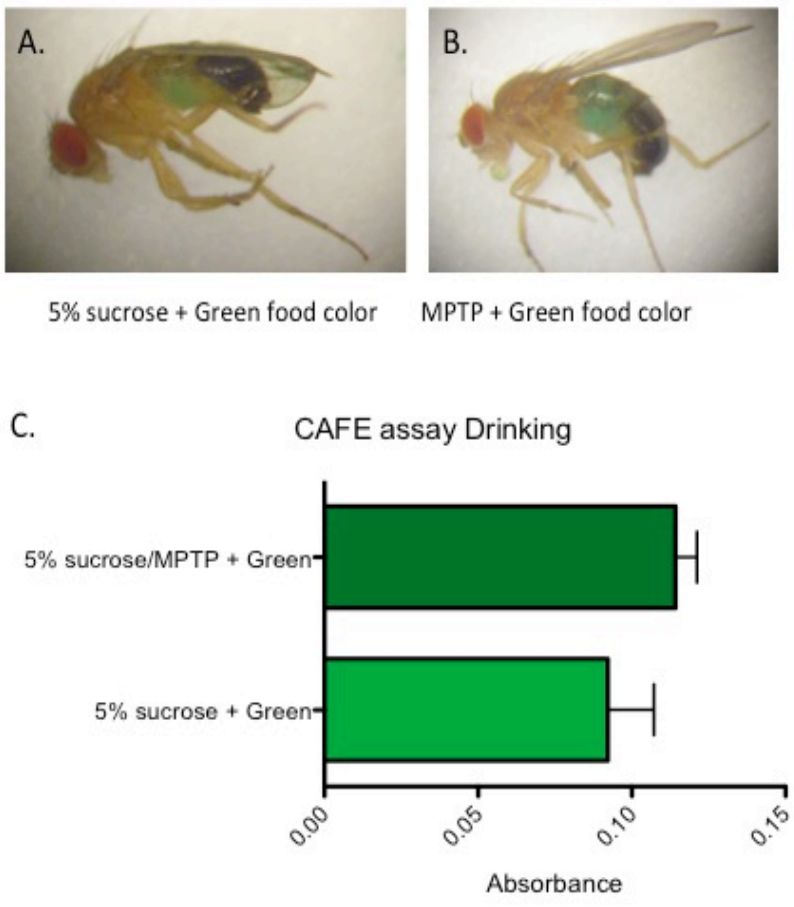


Figure 7. Comparison of CAFÉ consumption during exposure to MPTP and Sucrose

Comparison of 5% sucrose and MPTP (36 mM) and just 5% sucrose liquid was investigated to determine whether flies were not consuming less of the MPTP containing liquid. Green dye was added to each control and test liquid and photos were taken after CAFÉ assay exposure showing consumption of both

liquids (A and B). 100 flies were crushed in PBS and absorbance was measured at 630 nm in duplicate. There was no statistical difference found between the control (5% sucrose) and test liquid (MPTP with sucrose).

FIGURE 8

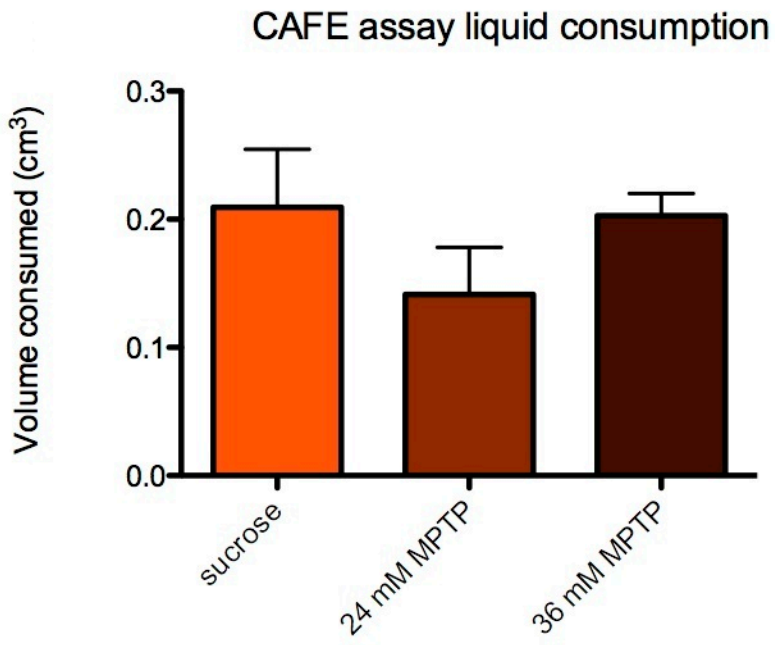


Figure 8. Volume consumption in capillary tubes during CAFÉ assay exposure

Further validation of CAFÉ assay exposure with MPTP for the two highest concentrations used of MPTP. No statistical difference was found between the control group and either of the exposures (24 mM & 36 mM) (n=5).

Assessment of Brain Dopamine Levels after Exposure to MPTP

In order to determine whether MPTP is toxic to DaN, flies where exposed to 3 mM, 6 mM, 12 mM, 24 mM, or 36 mM MPTP by the CAFÉ assay. Brain homogenates from the different exposure groups were analyzed via rp-HPLC-ECD to determine the amount of dopamine per fly brain (n=5). Figure 9 is a representative chromatogram for dopamine analysis indicating the peak for dopamine and the internal standard. Levels of dopamine in brain homogenates were obtained by comparing the response in the extract to responses obtained by known amounts of dopamine. In Figure 10, the amount of dopamine per fly brain for each concentration of MPTP is represented with 5% sucrose as control. We established that 5% sucrose was suitable for a control because no difference in dopamine levels was found when comparing to normal food 24-hour exposure (Figure 11).

FIGURE 9

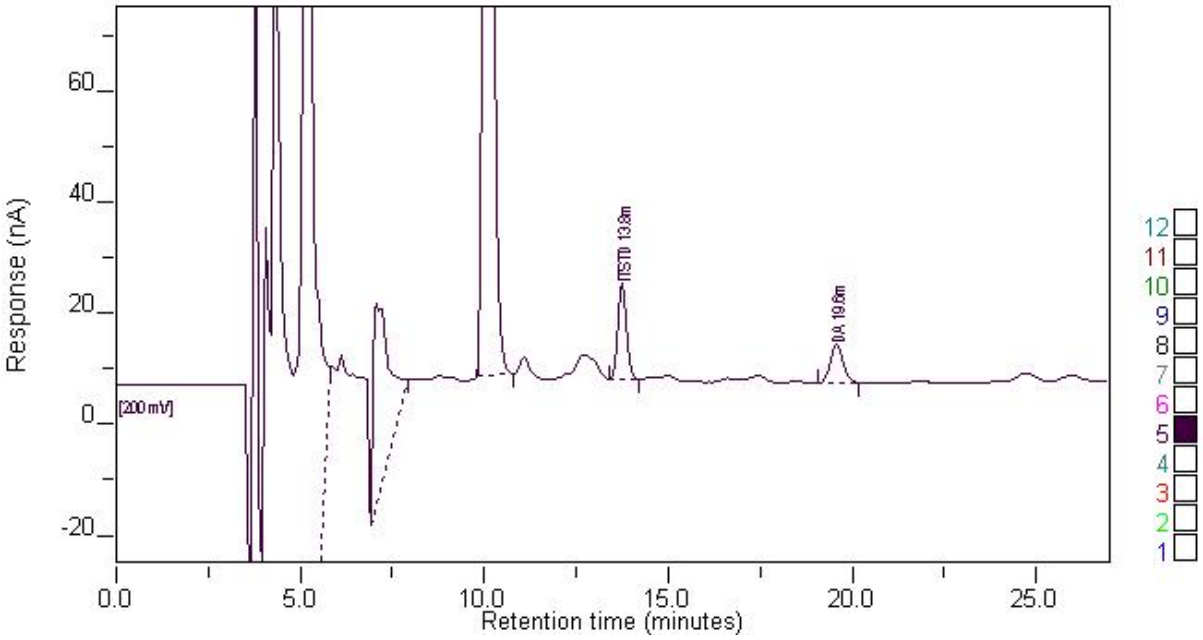


Figure 9. Chromatogram profile for Dopamine

Chromatographic profile for Dopamine and Internal Standard (DBA), detected in channels set at 300 mV.

FIGURE 10

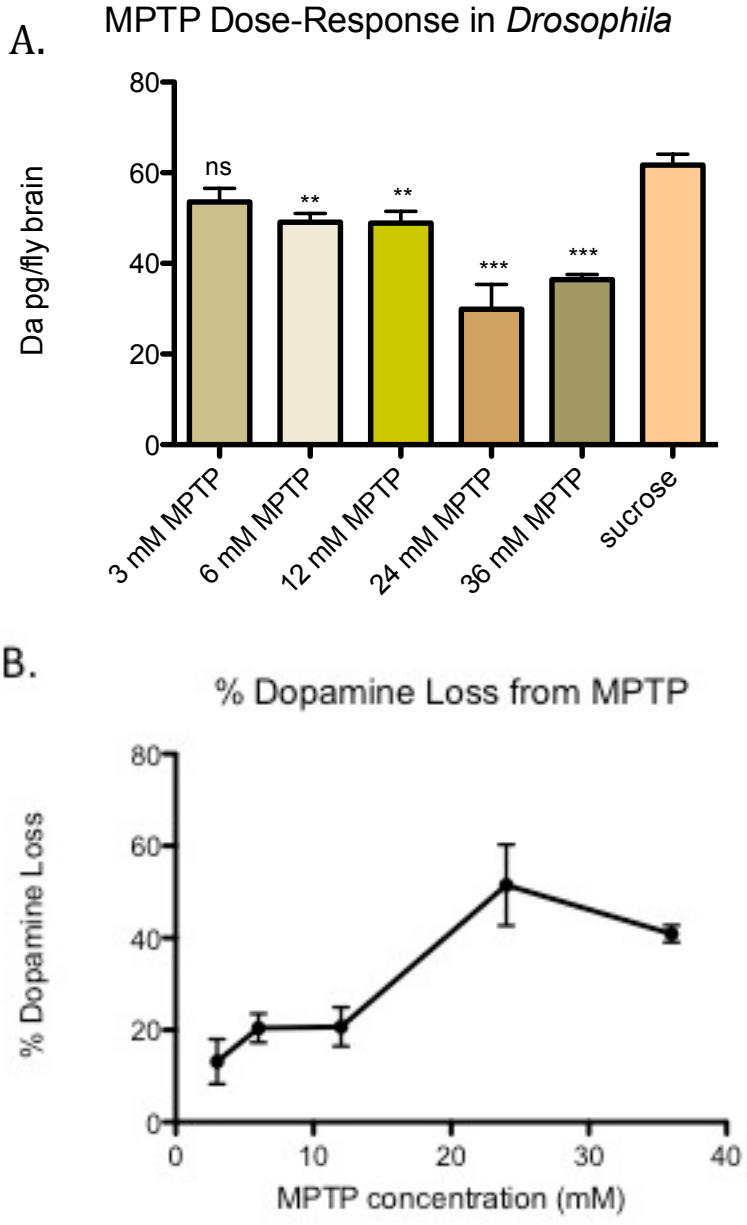


Figure 10. Dopamine levels in brain after exposure to MPTP

Levels of Dopamine represented as picogram (pg) DA per fly brain after CAFÉ assay exposure to 3 mM, 6 mM, 12 mM, 24 mM, and 36 mM MPTP in WT male flies compared to 5% sucrose as control (A). Although all groups exposed showed a reduction in DA compared to control, 36 mM and 24 mM

concentrations had the greatest significant difference. In (B), values are expressed as percent DA loss. The greatest percent loss was seen in 24 and 36 mM MPTP exposures. Data expressed as mean ±SEM (n=5 ***p< .005 **p<.05)

FIGURE 11

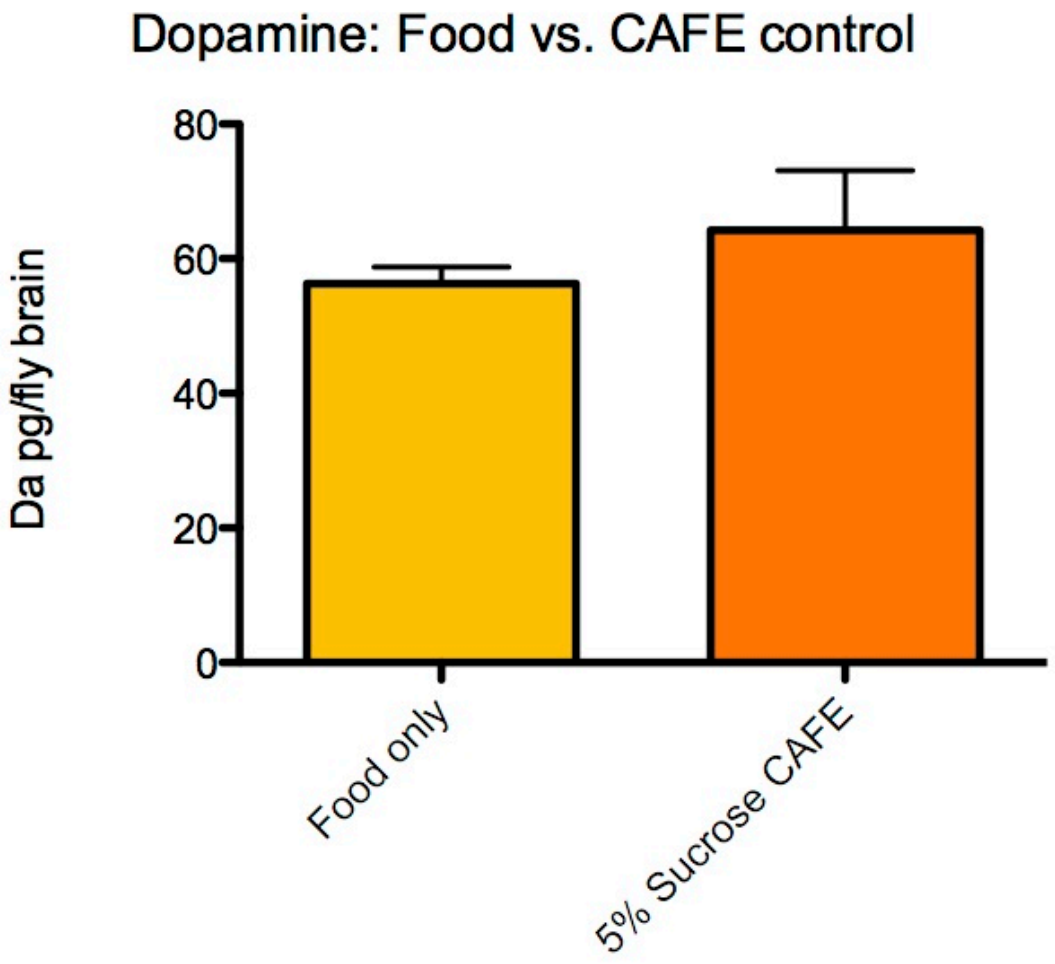


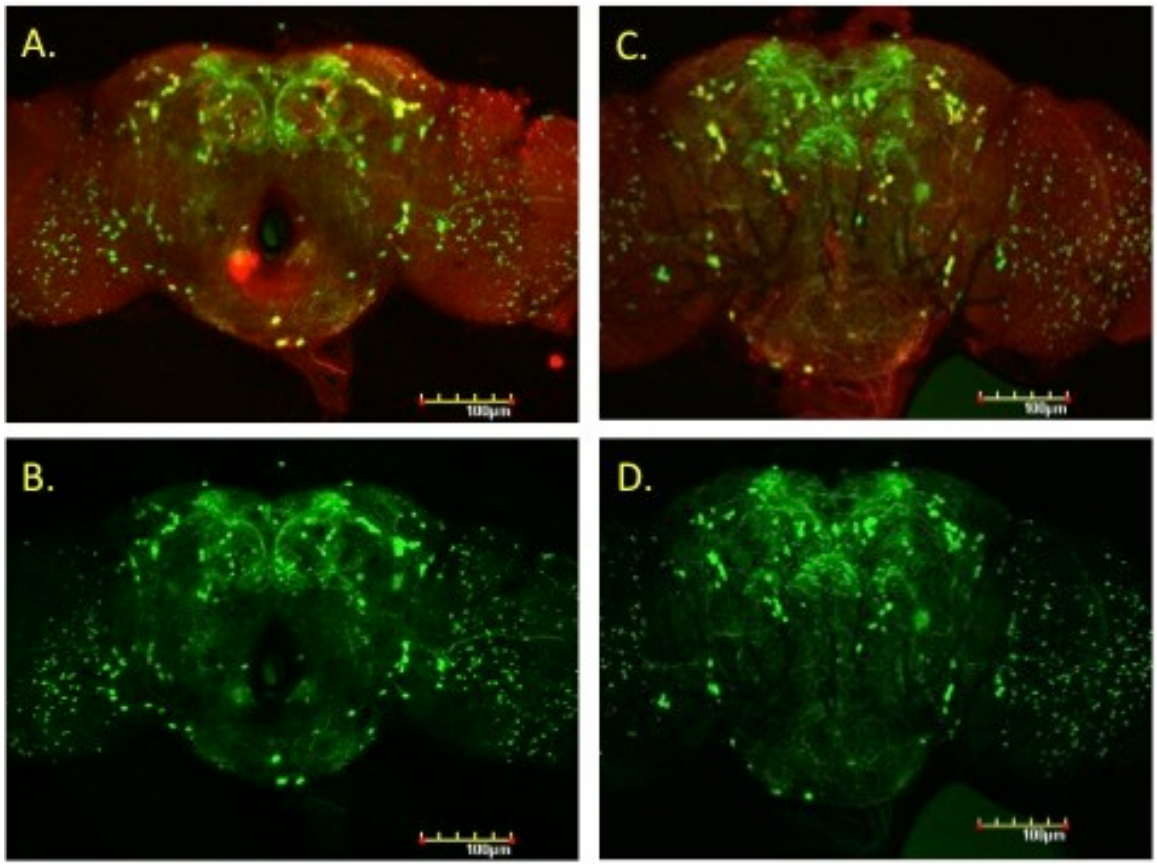
Figure 11. Dopamine levels from 5% sucrose 24-hour exposure compared to food control

Dopamine levels represented as % change when comparing CAFÉ assay 5% sucrose exposure vs. normal food control. No statistical difference was found.

Tyrosine-Hydroxylase labeling in *Drosophila* brain

To test whether changes in dopamine levels were due to loss of DaN, we performed immunohistochemistry for tyrosine hydroxylase (TH) in fly brains exposed to 24 mM MPTP. Figure 12A shows the confocal images taken from sucrose exposed or 24 mM exposed fly brains expressing TH by the Gal-4 driver and the quantification of TH-expressing neurons for each group.

FIGURE 12



Tyrosine Hydroxylase Positive Cells

Sucrose Exposed	24 mM MPTP Exposed
44	34
35	29
20	22
Average:	Average:
33	28.3

Figure 12. Tyrosine hydroxylase Immunohistochemistry after MPTP exposure

Flies brains expressing GFP labeled Tyrosine Hydroxylase (Green) by the Gal-4 UAS system compared with rabbit anti-Tyrosine hydroxylase antibody labeling (red). Neurons were counted in sucrose (A and B) control and compared to

MPTP exposed flies (C and D). The average of three separate brain images was calculated.

Behavior after exposure to MPTP

Because motor impairment is a direct symptom of PD and being present in other MPTP models, we employed the startle-induced negative geotaxis assay to assess whether there was impairment in climbing to MPTP exposed flies compared to untreated flies (Figure 13). Typically, flies without impairment would climb towards the light source after being startled to the bottom; therefore most flies would climb above 7cm in the conical tube.

FIGURE 13

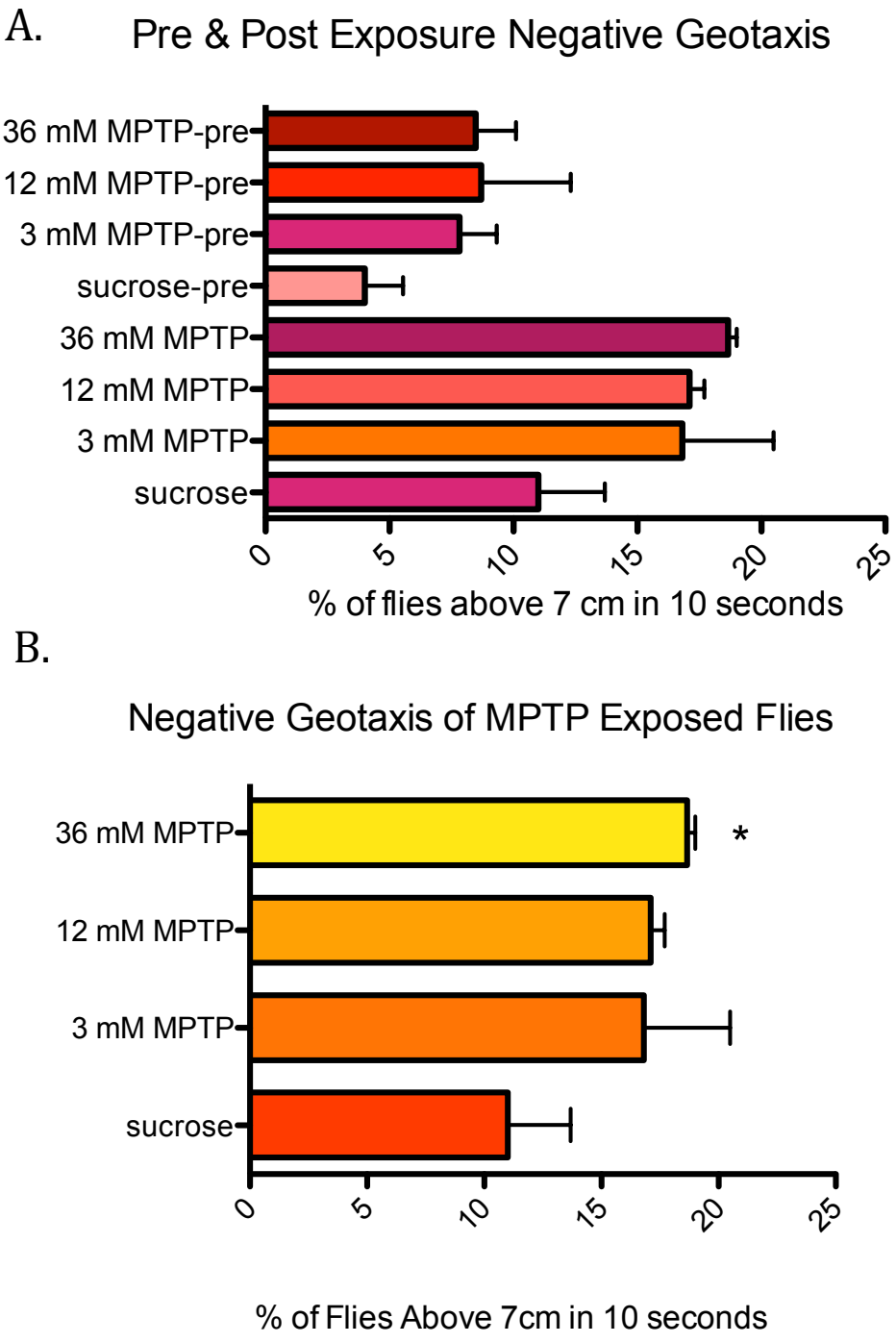


Figure 13. Startle-Induced Negative geotaxis of MPTP exposed flies

Behavior analysis of flies after MPTP exposure compared to sucrose control. Flies were counted before and after exposure. Percent flies above 7 cm was

determined by counting the number of flies that climbed above 7 cm to the total number of flies. Percent flies above 7 cm increased after exposure to MPTP for all exposure, including control when compared to pre-exposure groups (A). Only the 36 mM MPTP dose had a significant increase in climbing activity compared to control (B).

Discussion

The MPTP-toxin induced toxicological PD model has never been investigated in *Drosophila melanogaster*. Until now, there has not been a model method reported to expose flies to MPTP. This may be due to MPTP's exposure risk concern and precautions that are required when used in other animal models. The current toxin models (rotenone and paraquat) analyzed in *Drosophila* do not have a complete analysis of Parkinson-like assessments that extend beyond behavior and immunohistology, or both. Here, we show evidence of a reduction in dopamine, which is the underlying definition of PD. Having also looked at behavioral analysis, the data is good support for a reduction in dopamine in which geotaxis would be expected to decrease, but it should not be the only assessment in a model of PD. Similarly, the indirect analysis of tyrosine hydroxylase (the rate-limiting step of dopamine) is not as accurate to determine amount of dopamine in the brain.

Here, we hypothesize that *Drosophila* which has all the required biological components to metabolize MPTP and to accumulate MPP+ in dopamine neurons would be susceptible to MPTP(Daneman and Barres 2005). To reduce exposure to lab personnel, we employed the CAFÉ assay to expose *Drosophila* rather than food vial exposure (Ja et al 2007). Our initial results support the use of this approach, as no aversion to the sucrose solution containing MPTP was evident (Stafford et al. 2012). By using a visual indicator, in this case green food dye, we compared the consumption amount of sucrose control liquid vs. MPTP liquid at

the highest concentration used (Figure 6). We found no significant difference in liquid consumption, when comparing the absorbance of control and exposed flies (Figure 7). In fact, in this case there seemed to be a slight increase in absorbance of green dye in flies exposed to MPTP. In addition, we measured the volume consumed in the capillary tubes before and after the CAFÉ assay. There was also not a significant difference in the volume consumed based on an n=5 (Figure 8).

To establish the potential of the MPTP-fly model, groups of flies were exposed for 24 hours to increasing concentrations of MPTP (3, 6, 12, 24, or 36 mM) via the CAFÉ assay. Figure 9 indicates that brain dopamine levels were reduced in all treated groups, except 3mM, after MPTP, when compared to sucrose-exposed flies. We determined that 24 mM and 36 mM led to the largest amount of dopamine loss compared to control (Figure 10A). When shown as percent loss of dopamine (Figure 10B), 24 mM and 36 mM MPTP exposure leads to a 40-60% dopamine loss, replicating the loss of dopamine in early stages PD. We determined there was no significant difference in dopamine using the sucrose CAFÉ exposed flies compared to normal food exposed flies (Figure 11). This difference was a concern based on other feeding studies showing variances in transcription due to dietary restriction (Bruce et al. 2013) (Ding et al. 2014).

Tyrosine hydroxylase (TH), the rate-limiting step in the synthesis of dopamine, was visualized to see if there was a deficit in dopamine-containing neurons. After exposure to 24 mM MPTP, TH-GFP expressing neurons by the

Gal-4 driver system (green) and co-localized with an antibody to TH (red) were counted. It was expected that a reduction in TH would correlate with loss of dopamine as shown in similarly exposed flies seen in the HPLC analysis. The counts seen in the table below the images in Figure 12 show the number of TH co-localized neurons counted. It was found that the MPTP number was lower than the sucrose-exposed control, although this was only based on three images per group, therefore not enough for statistical analysis.

Analysis of behavior did not result with what was expected. We hypothesized that a decrease in dopamine from MPTP exposure would result in decreased climbing activity as measure by lower number of flies climbing above 7 cm (Figure 13). This hypothesis is based on other studies where drosophila were exposed to either paraquat or rotenone and analyzed for geotaxis (Sudati et al. 2013) (Jahromi et al. 2013). Instead, the opposite was observed where there was an increase in climbing activity corresponding with increasing exposure to MPTP. Chronic studies with MPTP need to be done to test whether this increased motor activity is due to a compensatory mechanism that won't be permanent.

Conclusions

In conclusion, the MPTP model in *Drosophila melanogaster* proposes to be a suitable model for future investigation of mechanisms of PD pathology. This model of exposure can be expanded for a wide range of uses for therapeutic intervention for idiopathic and familial inherited forms of PD.

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CHAPTER THREE: AIM TWO

Correlating the inhibition of mitochondrial Complex I with the loss of the dopaminergic system in *Drosophila melanogaster*: Measurement of mitochondrial complex I activity after dose exposure to MPTP

Abstract

Dysfunction of mitochondrial complex I has been set forth as a main hypothesis for loss of DaN in PD. There is reduction in complex I activity in PD patients and most used neurotoxins to model PD work by inhibiting mitochondrial complex I. Correlating an MPTP-induced death with the inhibition of mitochondrial complex I is useful for determining the effectiveness of the model as well as establishing the conservation of mechanisms across species. This chapter looks at the verification of MPTP-induced inhibition of mitochondrial complex I.

Introduction

Parkinson's disease is associated with a systemic defect in mitochondrial complex I activity. Animal models indicate that exposure to inhibitors of mitochondrial complex I, including pesticides, is sufficient to reproduce the features of PD, but genetic factors clearly modulate susceptibility. Complex I defects may result in oxidative stress and increase the susceptibility of neurons to excitotoxic death. In this way, environmental exposures and mitochondrial dysfunction may interact and result in neurodegeneration. One of the major discoveries linking mitochondrial complex I with PD was the understanding of the toxic mechanism associated with MPTP dopaminergic neuronal loss (Banerjee et al. 2009).

The MPTP dosing regimen has been used in many animal models (excluding *Drosophila*) and is integral in gathering more information on the involvement of mitochondria in PD pathology. MPTP after crossing the blood-brain barrier is metabolized to MPP+ by monoamine oxidase B and taken up by dopaminergic neurons (DaN) by the dopamine transporter. Inside DaNs, MPTP is accumulated in mitochondria, where it inhibits the oxidation of nicotinamide adenine dinucleotide (NAD) substrates by blocking complex I in the electron transport chain. This inhibited oxidation leads to a build-up of reactive oxygen species generating damage to macromolecules.

Evaluating the activity of mitochondrial complex I in an MPTP model is key to show the consistency of the model of PD and may allow for the identification of a window of susceptibility to test therapeutic interventions to evaluate other

environmental exposures that may play a role in sporadic PD. Additionally, establishing a timeline between complex I inhibition and susceptibility of DaN may offer major advantage to advance evaluations with PD familial genetics that are known to inhibit the mitochondria, such as: DJ-1, LRRK2, PINK-1, and Parkin (Lesage and Brice 2012).

Materials and Methods

Chemicals and Reagents

All reagents used in assessment of mitochondrial complex I activity were purchased from Sigma Aldrich (St Louis, MO).

Fly Stocks

D. melanogaster wild-type (Canton-S) was obtained from the Bloomington Stock center (Bloomington, IN). The flies were reared on agar medium (1%, w/v brewer's yeast; 2%, w/v sucrose; 1%, w/v agar; 0.08%, v/w Tegosept) at constant temperature and humidity (23°C; 60% relative humidity, respectively). The flies were reared in 16 x 100 mm vials containing approximately 5 mL of medium at constant temperature, humidity (60%) and under 12h dark/light cycle. All experiments were performed with the same WT Canton-S strain.

Fly Exposures

The capillary feeder method (CAFE) CAFÉ assay used for exposure was adapted from JA, et al. 2007. The exposure model used was similar with two chambers. The inner chamber, containing the flies, was composed of a 25 cm vial with perforated lid for air and water exchange from the outer chamber, a 1000 mL beaker with 20 cm distilled water, and additional holes for capillary tube insertion. Capillary tubes at 40 cm length from World Precision Instruments (Sarasota, FL) (cat. #4878) were filled by capillary action with liquid and inserted into the inner vial lid with an additional layer of mineral oil to prevent evaporation. All exposures (MPTP or sucrose control) were conducted for 24 hours in a 25°C room. After each 24-hour exposure, flies were placed back into isolation vials

containing normal food medium for 24 hours before analyses. All exposures were done on 5-10 day old males.

Protein Preparation

Mitochondrial and cytosolic proteins were prepared using the mitochondria isolation kit from Abcam (Cat# ab110169). Briefly, brain from approximately 100 5-10 day-old WT male flies exposed to MPTP or 5% sucrose were dissected and homogenized in ice cold isolation buffer containing protein inhibitor cocktail (Roche Cat# 04693159001) using a glass dounce homogenizer. Each homogenate was centrifuged at 1000xg at 4°C for 10 min. The supernatant was then transferred to a new tube, and centrifuged again at 12,000xg at 4°C for 15 min to separate cytosolic and mitochondria proteins. The supernatant were collected as the cytosolic protein sample, while the pellet was washed and re-suspended in 200µl isolation buffer as the mitochondria protein sample. Protein concentration was determined with the BCA protein Assay kit (ThermoFisher, Cat# 23225) according to the manufacturer's suggested protocol.

Mitochondrial Complex I Activity

Measurement of complex I activity was modeled after reagent concentrations used in (Farge 2002). Complex I activity was determined by following the oxidation of NADH at 340 nm (e=6220 M^{-1} cm^{-1}) using ubiquinone-1 (coenzyme Q10) as the electron acceptor. The assay buffer consisted of 35 mM NaH_2PO_4 pH 7.2, 5 mM $MgCl_2$, 2.5 mg/ml BSA, 2 mM KCN, 2 µg/ml antimycin A, 97.5 µM ubiquinone, 0.13 mM NADH and 50 µg mitochondrial proteins. Each sample and control was done in duplicate in a 96-well assay plate. Rotenone was added in

duplicate to each sample as control for complete mitochondrial complex I inhibition.

Statistical Analysis

All data were analyzed using Microsoft Excel and Prism 5 software. For comparison of multiple groups, ANOVA was used to determine significant differences between each group with significance of p-value >.05.

Results

Mitochondrial Complex I activity in the Drosophila head

To further explore the impact of MPTP on the dopaminergic system in *Drosophila*, we wanted to quantify the dose in which MPTP inhibits complex I of the electron transport chain. Three doses were chosen based on the dose-response curve and loss of dopamine as discussed in the previous chapter (chapter two). Figure 14, shows the results of the assessment of complex I to reduce NADH in the presence of coenzyme Q10 by the measurement of NADH absorbance. Each exposure group was done in duplicate and normalized to the background of NADH.

FIGURE 14

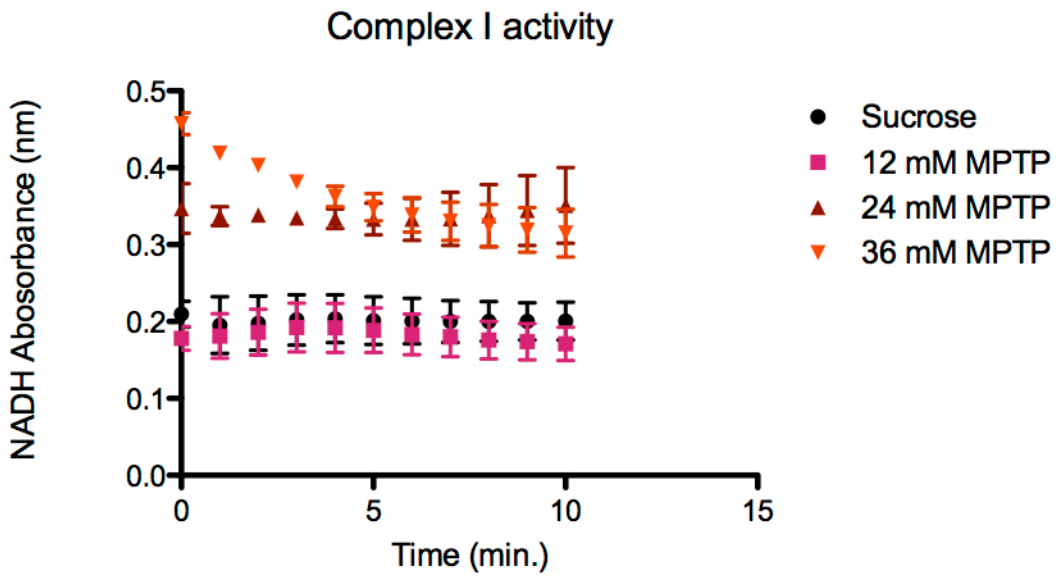

Figure 14. Mitochondrial Complex I activity after MPTP dose exposure

Absorbance of NADH oxidation was recorded by spectrophotometry at 430 nm. Each exposure group was performed in duplicate and measurements were taken every minute for 10 minutes. The higher absorbance indicates more NADH in the assay well, due to a lack of activity of mitochondrial complex I.

Discussion

Mitochondrial complex I is the target of MPTP-induced toxicity in dopaminergic neurons. Determining a MPTP dose-induced inhibition of mitochondrial complex I is important for examining a dose-susceptibility window. Here, we measured mitochondrial complex I activity with three doses of MPTP (12, 12, and 36 mM) in the drosophila MPTP model (Figure 14). This, to our knowledge, has not been done before in this model.

In Figure 14, we see a dose correlated loss of activity in complex I as measured by the absorbance of NADH. The higher absorbance of NADH is due to the lack of oxidation by complex I from its inhibition by MPTP (24 and 36 mM MPTP). Whereas, the control group (sucrose) and lower concentration (12 mM MPTP) exposure we do not see an inhibition of NADH as determined by a lower absorbance.

Conclusions

In conclusion, MPTP appears to inhibit complex I in the electron transport chain of the mitochondria that was dose-dependent. Together, the measured damage of MPTP, and using a dose above 24 mM proved to affect dopaminergic neurons in the brain of *Drosophila*.

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APPENDIX A:

Ogg1 susceptibility to age an MPTP in *Drosophila*

Oxidation of the guanine base is the most common form of oxidative stress-mediated damage to DNA leading to the formation and accumulation of the modified guanine lesion 8-hydroxy-2'-deoxyguanosine (oxo8dG). Accumulation of Oxo8dG has been linked to increased rate of mutations in dividing cells and transcription blockage in post-mitotic cells. High levels of oxo8dG are a common finding in affected brain areas in neurodegenerative diseases, particularly in the SN of PD patients. Levels of oxo8dG are maintained at bay by the activity of the DNA repair enzyme 8-oxoguanine glycosylase 1 (Ogg1). Mice lacking Ogg1 have an age-associated loss of the nigrostriatal pathway (Fernando Cardozo-Pelaez, Sanchez-Contreras, and Nevin 2012). This neuronal loss resembles parkinsonian-like pathology as seen by the specific age-dependent loss of nigral dopaminergic neurons as well as the accumulation of ubiquitin-positive inclusions in surviving neurons of the nigrostriatal region. Therefore, the results here examine the susceptibility of dopamine in Ogg1 knock-out ($Ogg1^{-/-}$) flies after aging and exposure to MPTP.

Summary of Method and Results

WT Canton-S flies or Ogg1 $Ogg1^{-/-}$ flies were exposed to 36 mM MPTP by the CAFÉ assay for 24 hours. After 24 hours, flies were changed to regular food and brains were collected another 24 hours after for dopamine analysis (Figure B). The dopamine levels were represented as percent loss compared to control, where $Ogg1^{-/-}$ showed a higher percent loss compared to WT, indicating they

may be more susceptible to MPTP. In Figure 15A, dopamine was quantified as picograms (pg) dopamine per brain. The same analysis was performed without exposure to MPTP, but flies were aged to 80 days.

This data is significant showing the drosophila may also have an age-associated loss of dopamine, similar to results shown in mice. Also, $Ogg1^{-/-}$ flies at a young age seem to be more susceptible to MPTP as expected.

References

Cardozo-Pelaez, Fernando, Monica Sanchez-Contreras, and Andrew B C Nevin. 2012. "Ogg1 Null Mice Exhibit Age-Associated Loss of the Nigrostriatal Pathway and Increased Sensitivity to MPTP." *Neurochemistry International* 61 (5): 721–30. doi:10.1016/j.neuint.2012.06.013.

FUNDING INFORMATION

This project was supported by the National Institutes of Health (NIA) grant number R01AG031184 and by NIGMS grand number P20RR017670. The content is solely the responsibility of the authors and does not necessarily represent the official views of the NIH or NIGMS.

FIGURE 15

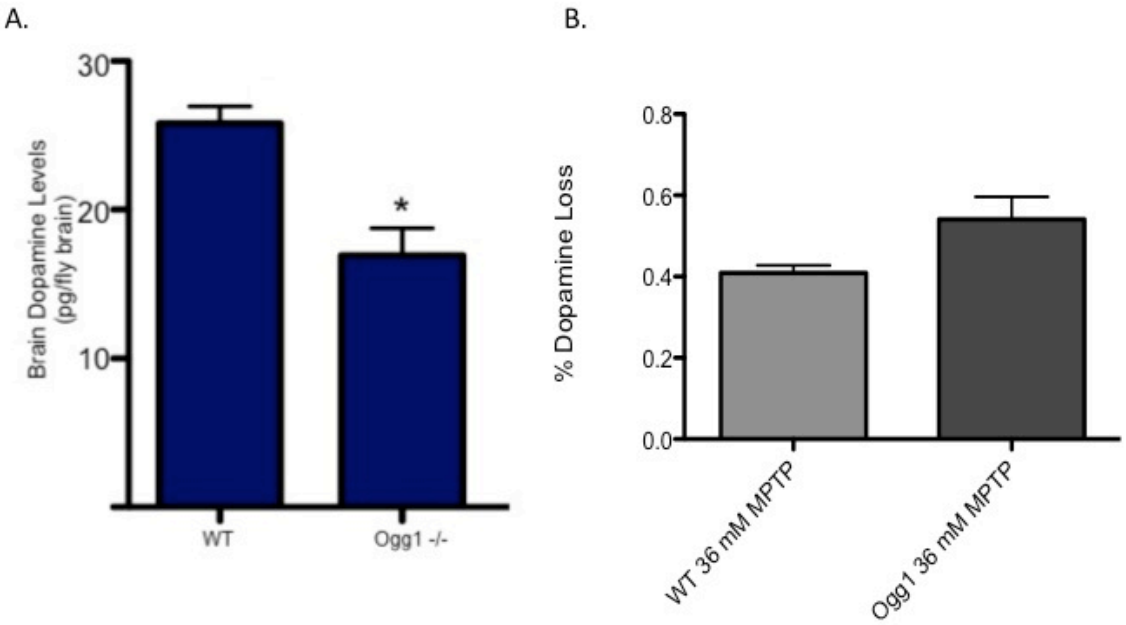

Figure 15. Ogg1 Dopamine Levels and susceptibility to MPTP

Dopamine level in brain of WT Canton-S flies compared to Ogg1 KO after aging for 80 days (A). The percent dopamine loss was also compared to young WT flies and Ogg1 KO at 36 mM MPTP for 24 hours.